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Biochimica et Biophysica Acta 1608 (2004) 97–103
BIOCHIMICA ET BIOPHYSICA ACTA
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Rhodoquinone reaction site of mitochondrial complex I, in parasitic helminth, *Ascaris suum*

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Received 8 July 2003; received in revised form 10 October 2003; accepted 17 October 2003

Abstract

The components and organization of the respiratory chain in helminth mitochondria vary remarkably depending upon the stage of the life cycle. Mitochondrial complex I in the parasitic helminth *Ascaris suum* uses ubiquinone-9 (UQ_9) and rhodoquinone-9 (RQ_9) under aerobic and anaerobic conditions, respectively. In this study, we investigated structural features of the quinone reduction site of *A. suum* complex I using a series of quinazoline-type inhibitors and also by the kinetic analysis of rhodoquinone-2 (RQ_2) and ubiquinone-2 (UQ_2) reduction. Structure–activity profiles of the inhibition by quinazolines were comparable, but not completely identical, between NADH-RQ_2 and NADH-UQ_2 oxidoreductase activities. However, the inhibitory mechanism of quinazolines was competitive and partially competitive against RQ_2 and UQ_2, respectively. The pH profiles of both activities differed remarkably; NADH-RQ_2 oxidoreductase activity showed an optimum pH at 7.6, whereas NADH-UQ_2 oxidoreductase activity showed two optima pH at 6.4 and 7.2. Our results indicate that although *A. suum* complex I uses both RQ_2 and UQ_2 as an electron acceptor, the manner of reaction (or binding) of the two quinones differs.

Keywords: *Ascaris suum*; Complex I; Rhodoquinone; Ubiquinone; Quinone reaction site

1. Introduction

Parasitic helminths have exploited a variety of energy transducing systems in their adaptation to peculiar habitats in their hosts [1–4]. The parasitic nematode, *Ascaris suum*, resides in the host small intestine where oxygen tensions are low, and has exploited a unique anaerobic respiratory chain to adapt to its microaerobic habitat, called the NADH-fumarate reductase system [5–8]. The system is part of the unique respiratory system of parasitic helminths [1–4] and is the terminal step in the phospho*enol*pyruvate carboxykinase-succinate pathway, which is found in many anaerobic organisms [9–11]. Electrons from NADH are accepted by rhodoquinone through complex I (NADH-rhodoquinone oxidoreductase), and then transferred to fumarate through complex II (rhodoquinol-fumarate reductase). The anaerobic electron transfer in complex I couples with proton transport across the innermitochondrial membrane, providing ATP even in the absence of oxygen. This system has been thought to be a good target for developing a new class of anthelmintics [12].

In the previous study [12], we reported that an anthelmintic compound, nafredin, selectively inhibits helminth complex I at concentrations in the order of nanomolar, but not rat liver mitochondrial complex I. Kinetic analysis revealed that the inhibition by nafredin is competitive against an exogenous short-chain rhodoquinone (*n*-decylrhodoquinone). These findings along with the fact that helminth complex I uses both rhodoquinone-9 (RQ_9) and ubiquinone-9 (UQ_9) as an electron acceptor suggest that the structural features of the quinone reduction site of helminth complex I may differ from that of mammalian complex I.

To probe the structural and functional characteristics of the terminal electron transfer step of bovine complex I,

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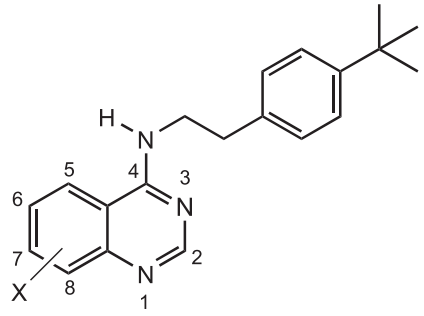
doi:10.1016/j.bbabio.2003.10.006



compound 1	X = H
compound 2	6-NH_2
compound 3	6-N_3
compound 4	6-NHCO(CH=CH_2)
compound 5	7-NH_2
compound 6	7-N_3
compound 7	8-OH
compound 8	8-OCH_3
compound 9	8-OCH_2CH_3
compound 10	8-$OCH(CH_3)_2$

Fig. 1. Structures of quinazoline derivatives.

potent and specific inhibitors have been shown to be useful tools [13–16]. Therefore, in a preliminary study, we screened a variety of bovine complex I inhibitors for *A. suum* complex I. Throughout the screening, we found that some quinazoline-type inhibitors (Fig. 1) are very effective against the enzyme and showed stronger inhibitory activity than nafredin. In this study, we characterized the quinone reduction site of *A. suum* complex I using a series of quinazolines with rhodoquinone-2 (RQ_2) or ubiquinone-2 (UQ_2) as an electron acceptor. Furthermore, we carried out detailed kinetic analyses of RQ_2 and UQ_2 reduction. Our results demonstrate that the manner of reaction (or binding) of UQ_2 and RQ_2 with helminth complex I differs.

2. Materials and methods

2.1. Chemicals and A. suum

UQ_2 was purchased from Sigma. RQ_2 was synthesized as described [17]. *A. suum* was obtained from a slaughter house, in Shibaura, Tokyo. Muscle of adult worm with cuticle was isolated and frozen at −80 °C. Compounds 1, 2 and 5 were synthesized as described previously [18]. The synthetic procedures of other quinazolines will be described elsewhere.

2.2. Preparation of A. suum mitochondria and complex I assay

The preparation of *A. suum* mitochondria from adult worms described previously [7] was improved as follows. Frozen muscles with cuticles were thawed at 4 °C overnight. After the separation of cuticles, muscles stored in 0.15 M KCl were minced with scissors. Minced muscle was suspended with the same volume (w/v) of Chappell-Perry buffer (50 mM Tris–HCl, pH 7.4, 0.1M KCl, 1 mM ATP, 5 mM $MgSO_4$ and 1 mM EDTA) and broken using a waring blender, the CELL MASTER CM-100, from Iuchi Co. (10,000 rpm for 1 min and 18,000 rpm for 1 min). Then the suspension was homogenized with a glass-teflon homogenizer (three strokes). After the addition of 10 volumes (w/w) of Chappell-Perry buffer, the homogenate was centrifuged at $750 \times g$ for 10 min. The supernatant was centrifuged again at $17\,000 \times g$ for 10 min. The pellets were collected and homogenized by grass-teflon homogenizer (three strokes). The homogenate was diluted with 10 volumes of Chappell-Perry buffer and centrifuged at $20\,000 \times g$ for 10 min. This operation was repeated several times. After the centrifugation, the pellets were collected as mitochondria and homogenized with a glass-teflon homogenizer (three strokes). Isolated mitochondria were frozen and stored at −80 °C. In most of the experiments, we did not use inside-out submitochondrial particles (SMP) because the amount of mitochondria isolated from the muscle of *A. suum* is very limited. Instead, mitochondrial preparations were sonicated shortly before use to obtain NADH-permeable mitochondrial preparations. These sonicated mitochondria oxidize externally added NADH.

Table 1
Inhibition of NADH-UQ_2 and NADH-RQ_2 oxidoreductase activity by quinazoline derivatives

Compound	IC_{50} (nM)			
	A. suum		Bovine	
	UQ_2	RQ_2	UQ_2	RQ_2[a]
1	1.3	3.8	25	75
2	0.8	1.8	10	13
3	100	150	48	95
4	130	480	1100	2200
5	72	300	250	1000
6	520	440	78	400
7	470	500	2900	11000
8	1.3	1.2	29	75
9	140	250	700	1000
10	600	430	1000	590

The IC_{50} value was defined as the molar concentration of the quinazoline derivatives that decreased NADH-UQ_2 and NADH-RQ_2 oxidoreductase activity with 50 μM UQ_2 and RQ_2, respectively, to 50% of the control activity.[b]

[a] Exceptionally, NADH-RQ_2 oxidoreductase activity of bovine SMP was carried out with 30 μM RQ_2, because substrate inhibition was observed at high concentration range of RQ_2.

[b] The values were averaged from two independent experiments.

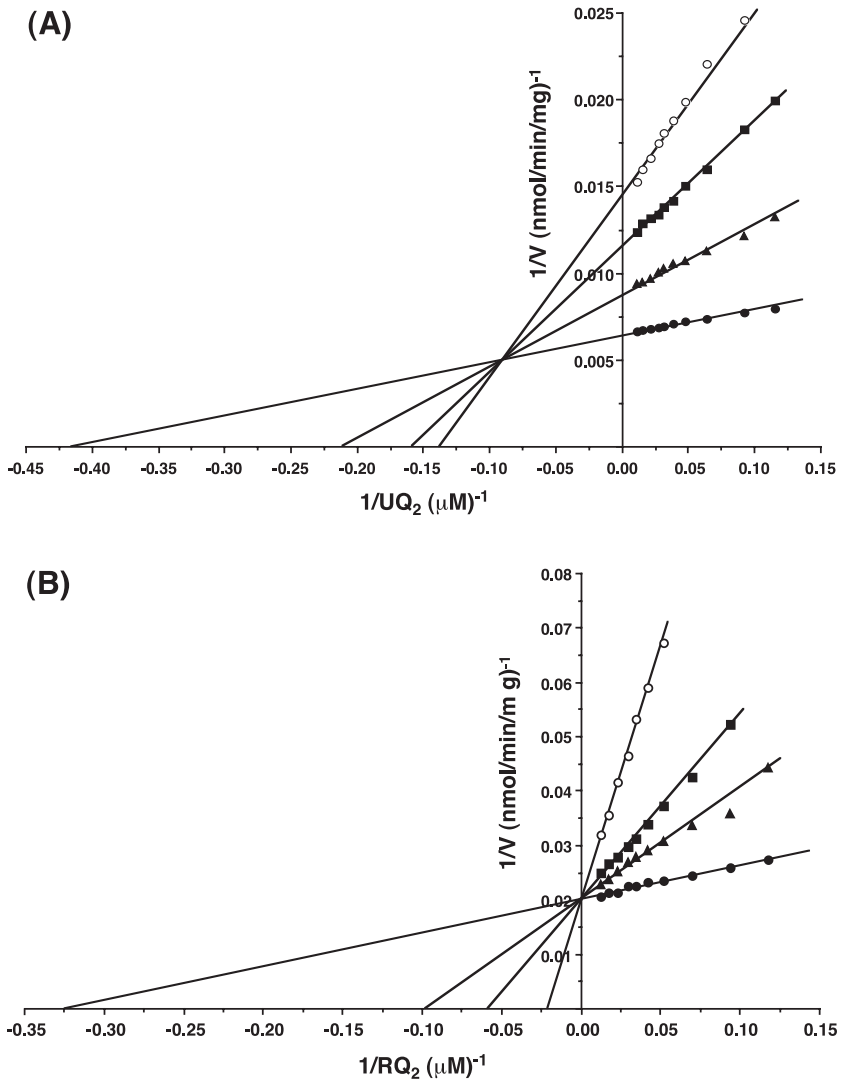


Fig. 2. Double-reciprocal plots of the inhibition of NADH-UQ_2 and NADH-RQ_2 oxidoreductase activities by compound 1 with various concentrations of UQ_2 (A) and RQ_2 (B), respectively. NADH-UQ_2 and NADH-RQ_2 oxidoreductase activities of *A. suum* adult mitochondria were measured in 50 mM potassium phosphate buffer, pH 7.2, containing (A) 0 nM (●), 0.3 nM (▲), 0.8 nM (■) and 1.5 nM compound 1 (○); (B) 0 nM (●), 0.3 nM (▲), 1 nM (■) and 4 nM compound 1 (○).

NADH-ubiquinone and NADH-rhodoquinone oxidoreductase assays, which were performed under aerobic conditions previously [12], were carried out under anaerobic condition as follows. The reason for the change in the assay conditions is described in the Section 3. These activities were measured spectrophotometrically by monitoring the oxidation of NADH at 340 nm ($\varepsilon = 6.2\ mM^{-1}\ cm^{-1}$) in a reaction mixture (total 1 ml) containing 50 mM potassium phosphate buffer, pH 7.2, 10 mM β-D-glucose, 20 units of glucose oxidase, 26 units of catalase, 200 μM NADH, 10–80 μM UQ_2/RQ_2 and enzyme solution. NADH-fumarate oxidoreductase activity was measured as described [12].

2.3. Preparation of bovine heart SMP and complex I assay

Preparation of bovine heart SMP and complex I assay were carried out as described previously [19]. However, to compare with *A. suum* complex I assay, bovine complex I assay was also carried out under anaerobic condition as follows: 10 mM β-D-glucose, 20 units of glucose oxidase and 26 units of catalase were added to original solution, and 2 mM KCN and 0.2 μM MOA-stilbene were omitted.

3. Results and discussion

3.1. Reactivity of A. suum mitochondrial complex I with UQ_2 and RQ_2

In the NADH-UQ_2 (or RQ_2) oxidoreductase assay for *A. suum* mitochondria, significant NADH oxidation was observed under aerobic conditions irrespective of the absence of exogenous quinone. This NADH oxidation could not be

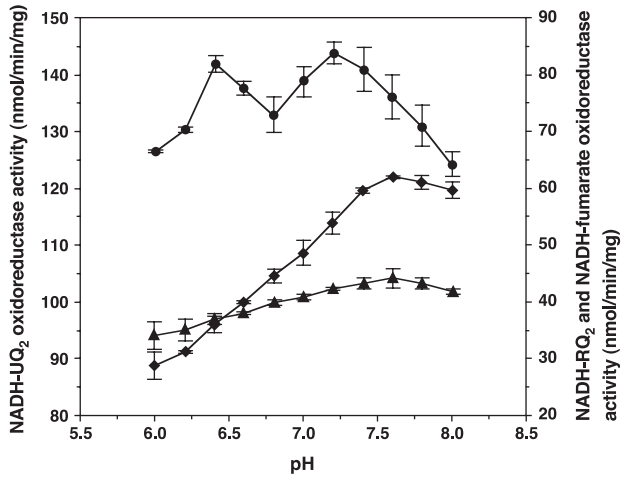


Fig. 3. The pH optima of NADH-UQ_2, NADH-RQ_2 and NADH-fumarate oxidoreductase activities. NADH-UQ_2, NADH-RQ_2 and NADH-fumarate oxidoreductase activities of *A. suum* adult mitochondria were measured with 50 μM UQ_2 (●) and RQ_2 (▲), and 5 mM fumarate (♦), respectively, in potassium phosphate buffer, between pH 6.0 and 8.0. The values reported represent the means ± S.D. of three independent measurements.

suppressed by any inhibitors of complexes III and IV such as antimycin A and KCN, and interferes with the accurate measurement of NADH-UQ_2 (or RQ_2) oxidoreductase activity. This phenomenon is probably due to direct electron transfer from endogenous rhodoquinol (RQ_9H_2) to molecular oxygen because of the high autoxidation rate of RQ_9H_2 to RQ_9. In fact, we could neither prepare nor store pure RQ_2H_2 using typical experimental procedures. Therefore, we carried out the assays anaerobically throughout this study as no NADH oxidation was observed under the anaerobic conditions described in the Section 2. [12]. Under these conditions, traces of NADH oxidation by mitochondria were linear at each concentration of UQ_2 and RQ_2, enabling accurate measurement of the enzyme activity. The averaged K_m and V_{max} values of NADH-UQ_2 and NADH-RQ_2 oxidoreductase activities are 2.4 ± 0.14 and 3.1 ± 0.02 μM, and 164 ± 9 and 52 ± 3 nmol NADH/min/mg of proteins, respectively. Although RQ_2 served as an electron acceptor of complex I in bovine SMP, substrate inhibition was observed at high concentration range of RQ_2, and the precise kinetic parameters could not be calculated.

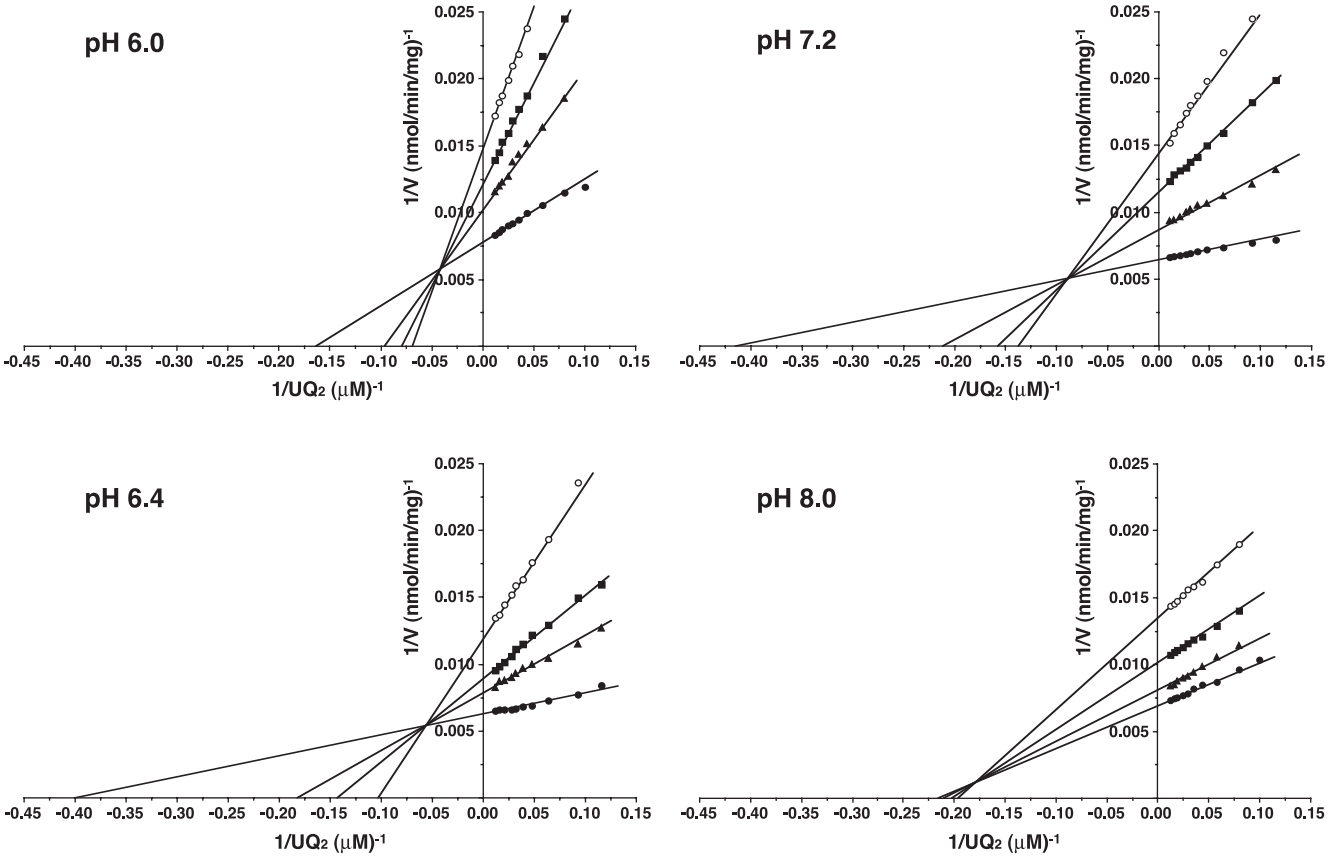


Fig. 4. Effect of pH on the mechanism of inhibition of NADH-UQ_2 oxidoreductase activity by compound 1. NADH-UQ_2 reductase activity of *A. suum* adult mitochondria was measured in 50 mM potassium phosphate buffer, pH 6.0 (A): 0 nM (●), 1.0 nM (▲), 1.5 nM (■) and 3.0 nM compound 1 (○); pH 6.4 (B): 0 nM (●), 0.5 nM (▲), 1.0 nM (■) and 3.0 nM compound 1 (○); pH 7.2 (C): 0 nM (●), 0.3 nM (▲), 0.8 nM (■) and 1.5 nM compound 1 (○); and pH 8.0 (D): 0 nM (●), 0.2 nM (▲), 0.4 nM (■) and 0.8 nM compound 1 (○).

3.2. Inhibition of A. suum complex I by quinazolines

Quinazoline-type inhibitors are potent inhibitors of bovine heart mitochondrial complex I [13,18]. In a preliminary study, we found that compound 1 (IC_{50} = 1.3 nM) is a very strong inhibitor of *A. suum* complex I superior to nafredin (IC_{50} = 10 nM), the most potent inhibitor of the enzyme reported so far [12]. To find an even more potent inhibitor, we tried to introduce a variety of substituents into the quinazoline ring and obtained the derivatives shown in Fig. 1. Although 6-amino and 8-methoxy derivatives elicited inhibition comparable to or slightly stronger than that of compound 1, the inhibitory potency, in general, drastically decreased even with slight structural modification. This result means that the structure of the inhibitor is strictly recognized by the enzyme (e.g. 8-NH_2 vs. 7-NH_2 and 8-MeO vs. 8-EtO). Thus, the remarkable structural dependency of the inhibition by quinazolines is useful to detect whether the interaction between the inhibitors and the enzyme is affected by the quinone used as an electron acceptor.

The structure–activity profiles for the inhibition by quinazolines were compared between NADH-UQ_2 and NADH-RQ_2 oxidoreductases with *A. suum* complex I. As is clear from Table 1, the profiles were comparable, but not completely identical, indicating that the enzyme recognizes the inhibitor in a similar way regardless of the electron acceptor. It should be realized, however, that this result does not necessarily mean that UQ_2 and RQ_2 react with complex I in an identical manner. One cannot rule out the possibility that if the binding sites of the quinazolines and quinones entirely differ, the inhibitory action (or binding) of quinazolines would not be affected by the quinones.

To better understand this point, we examined the inhibition by quinazolines with Lineweaver–Burk plots using UQ_2 or RQ_2 as an electron acceptor. As shown in Fig. 2, compound 1 inhibited complex I acting in a partially competitive manner against UQ_2. In contrast, compound 1 acted competitively against RQ_2. Similar results were observed for compounds 5 and 8 (data not shown). It is noteworthy that nafredin also shows a similar pattern of inhibition when UQ_2 or RQ_2 is used [12]. This finding suggests that the manner of reaction (or site) of UQ_2 and RQ_2 with the enzyme somewhat differs.

We also compared the structure–activity profiles for the inhibition by quinazolines between the two activities with bovine complex I (Table 1). The profiles were almost comparable, but not completely identical. The examination on

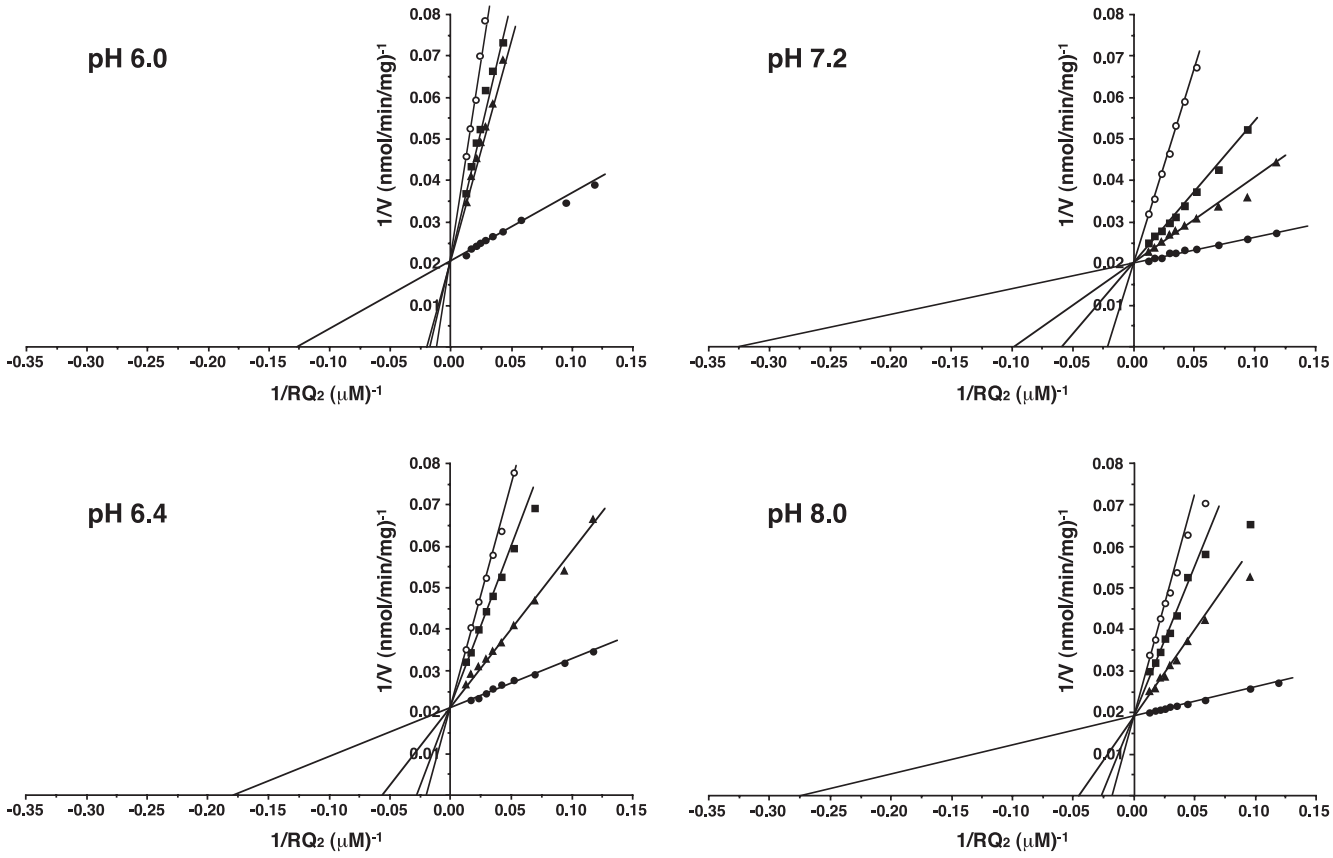


Fig. 5. Effect of pH on the mechanism of inhibition of NADH-RQ_2 oxidoreductase activity by compound 1. NADH-RQ_2 reductase activity of *A. suum* adult mitochondria was measured in 50 mM potassium phosphate buffer, pH 6.0 (A): 0 nM (●), 4.0 nM (▲), 10 nM (■) and 30 nM compound 1 (○); pH 6.4 (B): 0 nM (●), 0.7 nM (▲), 2.5 nM (■) and 10 nM compound 1 (○); pH 7.2 (C): 0 nM (●), 0.3 nM (▲), 1.0 nM (■) and 4.0 nM compound 1 (○); and pH 8.0 (D): 0 nM (●), 0.8 nM (▲), 1.5 nM (■) and 3.0 nM compound 1 (○).

inhibition kinetics for NADH-RQ_2 oxidoreductase activity was impractical because of significant substrate inhibition at high concentration range of RQ_2 as described above.

3.3. Quinone reduction site of *A. suum* complex I

To further characterize the quinone reduction with *A. suum* complex I, both NADH-UQ_2 and NADH-RQ_2 oxidoreductase activities were measured at various pHs ($6.0 < pH < 8.0$). As the optimum pH of glucose oxidase and catalase, which were contained in the reaction medium to keep conditions anaerobic, is about 7, we did not examine beyond this pH range. As shown in Fig. 3, the optimum pH of NADH-RQ_2 oxidoreductase activity was 7.6, which was comparable to that of NADH-fumarate reductase activity. Unexpectedly, NADH-UQ_2 oxidoreductase showed two optima at pH 6.4 and 7.2. Such a phenomenon has never been reported for other complex I enzymes as far as we know. In our experiments, bovine complex I showed optimum at pH 7.6 with UQ_2 and RQ_2 (data not shown). These results suggest not only that the manner of the reaction of UQ_2 and RQ_2 differs with *A. suun* complex I, but also that the reaction mechanism of UQ_2 varies depending upon pH. Therefore, we examined the pH-dependency of the inhibitory action of compound 1 for NADH-UQ_2 oxidoreductase activity (Fig. 4). In the pH range examined, the inhibitory mechanism remained partially competitive against UQ_2, although mixed-type inhibition kinetics varied with pH. Thus, the change in the reaction mechanism of UQ_2 may be negligibly small, if there is any. The competitive inhibition by compound 1 against RQ_2 for NADH-RQ_2 oxidoreductase was not affected by pH changes (Fig. 5).

The components and organization of the respiratory chain in helminth mitochondria vary widely depending upon the stage of the life cycle and habitat [1–4]. It is therefore interesting to elucidate whether the subunit composition of respiratory enzymes and reaction manner of the quinones (UQ and RQ) change with life stage. The present study for the first time demonstrates that manners of reaction (or binding) of UQ_2 and RQ_2 with *A. suum* complex I are not identical, although the structural difference between the two quinones is rather slight (i.e. 3-MeO vs. 3-NH_2). Nevertheless, we do not consider the reaction (or binding) sites of the two quinones to be quite different since the structure–activity relationship of the inhibitory action of quinazolines is comparable, though not completely identical, between NADH-UQ_2 and NADH-RQ_2 oxidoreductase activities, and RQ_2 and UQ_2 compete and at least partially compete with quinazolines, respectively.

It remains to be elucidated whether the molecular architecture of *A. suum* complex I for quinone reduction varies with the stage of the life cycle. In this regard, it is of interest to note that the subunit composition of *A. suum* complex II changes during the life cycle [20]. The study on *A. suum* complex I is now under investigation in our laboratories.

Acknowledgements

We would like to thank Dr. S. Takamiya (Department of Parasitology, School of Medicine, Juntendo University, Japan) and N. Shinjo for useful discussions. This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Science, Culture and Spot, Japan (13226015 and 13854011).

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